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Initiation of olfactory placode development and neurogenesis is blocked in mice lacking both *Six1* and *Six4*

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Abstract

Mouse olfactory epithelium (OE) originates from ectodermally derived placode, the olfactory placode that arises at the anterior end of the neural plate. Tissue grafting and recombination experiments suggest that the placode is derived from a common preplacodal domain around the neural plate and its development is directed by signals arising from the underlying mesoderm and adjacent neuroectoderm. In mice, loss of *Six1* affects OE morphogenesis but not placode formation. We show here that embryos lacking both *Six1* and *Six4* failed to form the olfactory placode but the preplacodal region appeared to be specified as judged by the expression of *Eya2*, which marks the common preplacodal domain, suggesting a synergistic requirement of *Six1* and *Six4* in patterning the preplacodal ectoderm to a morphologic placode. Our results show that *Six1* and *Six4* are coexpressed in the preplacodal ectoderm from E8.0. In the olfactory pit, *Six4* expression was observed in the peripheral precursors that overlap with *Mash1*-expressing cells, the early committed neuronal lineage. In contrast, *Six1* is highly distributed in the peripheral regions where stem cells reside at E10.5 and it overlaps with *Sox2* expression. Both genes are expressed in the basal and apical neuronal progenitors in the OE. Analyses of *Six1;Six4* double mutant embryos demonstrated that the slightly thickened epithelium observed in the mutant was not induced for neuronal development. In contrast, in *Six1*$^{-/-}$ embryos, all neuronal lineage markers were initially expressed but the pattern of their expression was altered. Although very few, the pioneer neurons were initially present in the *Six1* mutant OE. However, neurogenesis ceased by E12.5 due to markedly increased cell apoptosis and reduced proliferation, thus defining the cellular defects occurring in *Six1*$^{-/-}$ OE that have not been previously observed. Our findings demonstrate that *Six1/4* function at the top of early events controlling olfactory placode formation and neuronal development. Our analyses show that the threshold of Six1/4 may be crucial for the expression of olfactory specific genes and that Six1 and Six4 may act synergistically to mediate olfactory placode specification and patterning through Fgf and Bmp signaling pathways.

Keywords

Six1; Six4; Eya; Preplacodal region; Olfactory fate; Neurogenesis; Sox2; Fgf; Bmp4

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.10.039.

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Introduction

Mouse olfactory epithelium (OE) originates from ectodermally derived neurogenic placode, the olfactory placode that arises at the anterior end of the neural plate. Transplantation studies in amphibia and avians have established that the region of surface ectoderm competent to form an olfactory fate is initially quite large (Schlosser, 2006; Streit, 2007). As development proceeds, the region of olfactory competency becomes progressively restricted and the placodal tissue adjacent to the anterior end of the neural plate becomes specified for an olfactory fate. Once the olfactory placode is specified, it invaginates to form the olfactory/ nasal pit at around E10.5, and then continues to deepen and form more recesses as development proceeds. The OE is a simple sensory epithelium that contains two populations of progenitor cells: a population of neuronal precursors that divide apically and another population of progenitor cells that settle on the basal side of the OE where they continue to divide before differentiating into olfactory sensory neurons (OSNs) (Smart, 1971; Caggiano et al., 1994). The olfactory neurogenesis is under the control of a temporal series of intrinsic transcriptional cascades (Cau et al., 2002; Nicolay et al., 2006). The basic helix–loop–helix (bHLH) transcription factors, Mash1, neurogenins (Ngns), NeuroD and others that play a central role in the determination of muscle and nerve cells (Weintraub,1993; Jan and Jan, 1994), are sequentially expressed in the olfactory neural lineage (Cau et al., 1997). Recent studies in vitro and in vivo have demonstrated that during OSN development, Sox2-expressing stem cells located at the basal OE undergo asymmetric cell divisions to self renew and generate transient amplifying progenitors (Mash1-positive) and subsequently Ngn1-positive immediate neuronal precursors (Beites et al., 2005; Calof et al. 2002; Cau et al., 2002). The Ngn1-positive immediate precursors divide to differentiate into OSNs that express characteristic markers including neural cell adhesion molecule (NCAM), growth associated protein (Gap43), olfactory marker protein (OMP) and olfactory-specific genes essential for odorant transduction (Bakalyar and Reed, 1990; Brunet et al., 1996; Calof and Chikaraishi, 1989; Jones and Reed, 1989; Kawauchi et al., 2004). In addition to these transcription factors, several signaling pathways, including fibroblast growth factors (Fgfs) and transforming growth factors (Tgfs), are essential for normal development of the OE (Kawauchi et al., 2004). The Fgf signaling has been shown to play multiple roles during olfactory development, including an early role in placode induction (Bailey et al., 2006) and later roles in OE patterning (LaMantia et al., 2000), cell proliferation and survival (Kawauchi et al., 2004; Kawauchi et al., 2005). Although these studies have started to define specific transcription factors and signaling molecules in OE neurogenesis, how the placodal ectodermal cells are induced to differentiate into neuronal cells and the molecular mechanisms that direct their development along this pathway are not well established.

The murine Six gene family, homologous to *sine oculis* (so), which is required for normal eye development in *Drosophila* (Serikaku and O'Tousa,1994), is composed of six members (*Six1–6*) and encodes a Six domain (SD) and a Six-type homeodomain (HD), both are required for specific DNA binding and cooperative interactions with co-factors. In *Drosophila, so* acts in a molecular network that involves the fly *Pax6* gene *eyeless* (*ey*), *eyes absent* (*eya*) and *dachshund* (*dach*) to regulate eye development (reviewed in Treisman, 1999). In *Drosophila* eye development, *so* functions downstream of *eya* and their gene

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products participate in protein–protein interaction (Pignoni et al., 1997). Genetic studies in the mouse have shown that the Eya–So regulatory pathway controls early differentiation and survival of placodally derived cranial neurons (Zou et al., 2004; Zheng et al., 2003). For example, in the otic placode, Eya1 and Six1 are necessary for normal maintenance of inner ear neurogenesis (Xu et al., 1999; Zheng et al., 2003; Zou et al., 2004), and both genes act as critical determination factors in acquiring both neuronal fate and neuronal subtype identity from epibranchial placodal progenitors (Zou et al., 2004). Eya1 and Six1 appear to function upstream of bHLH genes, as *Eya1*$^{-/-}$ or *Six1*$^{-/-}$ placodal cells failed to express the *Ngn2*-regulatory neuronal differentiation program that is required for the production of epibranchial neuronal progenitors (Zou et al., 2004). However, the specific role of Eya and Six genes in such regulatory cascades has not yet been systematically addressed.

During the development of the cranial placodes, *Six1* and *Six4* are the preplacodal markers in chick, Xenopus and zebrafish (Bailey and Streit, 2006; Baker and Bronner-Fraser, 2001; Schlosser and Ahrens, 2004), while *Eya1* marks the Xenopus preplacodal region and *Eya2* is expressed in the chick and zebrafish preplacodal region (Mishima and Tomarev, 1998; Schlosser, 2006). It is still unclear whether these genes are expressed in the preplacodal region in mouse embryos, although *Six1/4* and *Eya1/2* are expressed in the olfactory placode (Xu et al., 1997a,b). A recent study has shown that loss of *Six1* function results in defective OE neurogenesis (Ikeda et al., 2007). However, the functional roles of *Eya* and *Six* genes in the regulation of olfactory development and neurogenesis are still poorly understood. It is also unclear whether *Six1* and *Six4* may compensate for each other's role in early placode development, as *Six4* mutant mice do not display an embryonic phenotype (Ozaki et al., 2001).

In this study, we set out to establish the respective functions of *Six* genes in the OE by analyzing mice carrying null mutations in *Six1* and *Six4*. In *Six1;Six4* double mutant embryos, the olfactory placode failed to form and the initiation of olfactory development was blocked. Both *Six1* and *Six4* are expressed in the placodal ectoderm at around E8.5. Interestingly, in the olfactory pit, *Six4*-expressing cells appear to mark the earliest committed neuronal progenitors in the peripheral region, while *Six1* is strongly expressed in the peripheral stem cells and its expression overlaps with *Sox2*. In the developing OE, both genes are expressed in basal and apical progenitors. Analysis of *Six1;Six4* double mutant mice demonstrated that neuronal development was not initiated in the presumptive olfactory ectoderm. In contrast, analysis of *Six1*$^{-/-}$ OE demonstrated that all neuronal lineages, including pioneer neurons, were initially present but the pattern of neurogenesis was altered as judged by the spatial expression of neuronal lineage-specific markers. However, neurogenesis ceased by E12.5 due to markedly increased cell apoptosis and reduced proliferation, thus defining the cellular defects occurring in *Six1*$^{-/-}$ OE that have not been previously observed. Together, our results demonstrate the requirement of *Six1* and *Six4* in the initial establishment of olfactory placode and neuronal cell lineage and OE patterning.

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Materials and methods

Mice and genotyping

Six1$^{+/-}$(*Six1*$^{lacZ/+}$) and *Six1*$^{+/-}$;*Six4*$^{+/-}$mutant mice in C57BL/6J strain were used for this study. MPI-II embryonic stem (ES) cells were used for generating the *Six1* mutant mice (Laclef et al., 2003). *Six4* targeting construct was electroporated into *Six1-lacZ* embryonic stem cells to generate *Six1*$^{+/-}$;*Six4*$^{+/-}$double heterozygous mice (Grifone et al., 2005). Genotyping for these mice was previously described (Xu et al., 2002; Laclef et al., 2003; Grifone et al., 2005).

X-gal staining and in situ hybridization

Embryos were fixed and processed using standard procedures. X-gal staining was performed as previously described (Zheng et al., 2003).

Whole-mount and section in situ hybridization were carried out according to standard procedures with digoxigenin-labeled riboprobes specific for *Eya1, Eya2, Six1, Six4, Sox2, Mash1, Ngn1, NeuroD, Lhx2, Ebf1, Pdh1, Gap43, Hes5, Fgf3, Fgf8, Fgf10, Bmp4* and *Dlx5*. We used 5 embryos for each genotype at each stage for each probe, and the staining was consistent in each embryo.

TUNEL and BrdU-labeling assays

TUNEL assay was performed as described (Xu et al., 1999). To examine cell proliferation, time pregnant female mice were injected i. p. twice at 2-hour intervals with 5-bromodeoxyuridine (BrdU; Sigma) in PBS at 100 mg/kg and processed as described (Zheng et al., 2003). Two hours after second injection, embryos were dissected, fixed and paraffin wax embedded. Paraffin wax embedded sections of 8 μm were prepared and denatured with 4N HCl for 1 h at 37 °C. Mouse anti-BrdU monoclonal antibody and goat anti-mouse IgG coupled with HRP or Cy3 were used for detection.

Result

Six1 and Six4 are expressed early during olfactory placode development and neurogenesis

To test how Six1 and Six4 might act to regulate olfactory placode development and neurogenesis, we first performed expression studies from stages of initial neurogenesis in the olfactory placode of mouse embryos to established/regenerative neurogenesis in the mature OE (Beites et al., 2005). *Six4* is expressed in a wide domain of the ectoderm in the presumptive olfactory region (Fig. 1A) and in the thickened olfactory placode (data not shown). It has been suggested that the neurogenesis in the OE proceeds in a periphery-to-center fashion, with early committed progenitors at the peripheral and terminally differentiated OSNs in the center of olfactory pit (Cau et al., 1997). *Six4* expression was observed in the peripheral precursors of the pit (arrows, Fig. 1B), which shows overlapping pattern with *Mash1*-expressing cells. At around E12.5–13.5, the OE begins to become organized with OSN progenitors progressively localized to the base of the epithelium, postmitotic neurons to an intermediate zone and apical progenitors. The intermediate layer

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contains neurons exhibiting a basal to apical maturation gradient such that mature OSNs are found closer to the outer apical layer, which also contains the supporting sustentacular cells that differentiate at around E15.5 (Smart, 1971). Interestingly, *Six4* expression became progressively restricted to the apical and basal progenitors at E12.5–14.5 (Figs. 1C, D and data not shown).

Similarly to *Six4, Six1* is strongly expressed in a wide ectodermal region in the presumptive olfactory domain and its expression becomes restricted to the olfactory placode at E9.5 (Fig. 1E). As the inserted *lacZ* transgene displayed an expression pattern identical to the *Six1* mRNA distribution obtained by in situ hybridization (Figs. 1E, I), we analyzed the expression of *Six1* gene during OE development in $Six1^{lacZ}$ heterozygotes by staining for *β*-galactosidase activity. At E10.25, *Six1-lacZ* expression was observed throughout the entire neuroepithelium of the invaginating placode (Fig. 1F). Shorter X-gal staining at E10.5 revealed that *Six1-lacZ* is highly distributed in the peripheral precursors of the pit (arrows, Fig. 1J). In contrast, only a subpopulation of the cells in the center of the pit expresses *Six1* (Fig. 1J). As *Six1* expression was not detected in the terminally differentiated OSNs, including the pioneer neurons that normally migrate away from the center of the placode at these early stages (data not shown), the *Six1*-positive cells located at the center of the placode are likely to be the *Ngn1*-positive proliferating immediate neuronal precursors. After E10.5, *Six1* expression became progressively restricted to the apical and basal progenitors at E12.5–14.5 (Figs. 1G, H, K, L). The expression pattern of *Six1* and *Six4* suggests that these genes may be essential for olfactory placode development. Their expression domain may mark early neuroepithelial stem cells as well as OE neuronal stem cells once the definitive OE structures have been established.

Six1 and Six4 act synergistically to control the formation of olfactory placodes

Previous work described that $Six1^{-/-}$ mice exhibited abnormal neurogenesis in the OE (Ikeda et al., 2007), while $Six4^{-/-}$ mice appeared normal (Ozaki et al., 2001). To examine whether *Six1* and *Six4* play redundant roles in olfactory placode development, we compared the morphological, molecular and genetic consequences for the olfactory development in mice lacking *Six1* alone and both *Six1* and *Six4* from E9.5 to 17.5. Histological sections of the nasal region of $Six1^{-/-}$ embryos revealed that the placode was formed and invaginated to form the pit at E10.5 (Fig. 2E), but the OE was noticeably thinner at these stages when compared with controls (Fig. 2A). Strikingly, the OE failed to deepen and form recesses and degenerated completely by E14.5 (Figs. 2F–H, compare with 2B–D). In addition, it failed to give rise to the VNO (Fig. 2H and data not shown). However, the nasal cartilage and septum were present in the mutant (Figs. 2G, H).

In contrast, the double mutant embryos revealed slightly thickened ectoderm at the position where normally forms the olfactory placode (arrow, Fig. 2I) and this ectoderm failed to invaginate to form the olfactory pit (Figs. 2J, K). This phenotype is bilateral in 12 embryos examined. In addition, no nasal septum is formed in the double mutant (Fig. 2K). Together, our results indicate that both *Six1* and *Six4* act synergistically to regulate the initiation of olfactory development, which arrested at the stage of placode formation in double mutant embryos, while *Six1* alone is essential for normal OE morphogenesis.

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Analysis of Six1/4 and Eya1/2 expression in the placodal ectoderm in the mouse embryos and the establishment of the preplacodal region is unaffected in Six1;Six4 double mutant embryos

To determine the stage at which olfactory abnormalities is initiated in the mutants, embryos were harvested at progressively earlier times and olfactory development was analyzed by marker gene analysis. Previous studies have shown that *Six1* and *Six4* are the preplacodal markers in *Xenopus*, chick, and zebrafish (Bailey and Streit, 2006; Baker and Bronner-Fraser, 2001; Schlosser and Ahrens, 2004), while *Eya1* marks the *Xenopus* preplacodal region and *Eya2* is expressed in the chick and zebrafish preplacodal region respectively (Mishima and Tomarev, 1998; Schlosser, 2006; Ishihara et al., 2008). However, it is unclear whether these genes are expressed in the preplacodal domain in the mouse embryos. In addition, the functional role of these genes in the preplacodal region is not established. As a first step to understanding their role in placode development, we sought to establish the expression profile of these genes in the placodal ectoderm during early mouse development. In situ hybridization and X-gal staining revealed that *Six1* is expressed in the preplacodal region (Figs. 3A, B) and its expression is maintained in individual placodes (Fig. 3C). Similarly, *Six4* is expressed strongly in the preplacodal region at E8.0 (Fig. 3D) and in the presumptive olfactory ectoderm at E9.0 (Fig. 3E). In contrast, *Eya1* expression in the placodal ectoderm was undetectable before E8.75 (Figs. 3F, G) but became detectable in the presumptive olfactory ectoderm at around E8.75–9.0 (Fig. 3H). By E10.5, X-gal staining of *Eya1*^{lacZ} allele (Zou et al., 2008) revealed that it is expressed in the progenitors located at the center of the pit (arrow, Fig. 3I), differing from the domains of *Six1/4* expression (Fig. 1). In contrast to *Eya1, Eya2* expression was strongly detected in the preplacodal region from early stages (Fig. 3J) and in the presumptive olfactory ectoderm (Fig. 3K), similarly to that of *Six1* and *Six4*. Interestingly, similar to *Six1, Eya2* expression was observed strongly in the peripheral stem cell progenitors and weakly in the center of the pit by both in situ hybridization (data not shown) and X-gal staining of the *Eya2*^{lacZ} knockin allele (Fig. 3M), which recaptured the endogenous gene expression (B. Chen, E-H. Kim and P-X. Xu, manuscript in preparation). These results indicate that during early placode formation, *Six1/4* together with *Eya2* appear to be expressed earlier than *Eya1* in the ectoderm but all five genes are expressed in the olfactory ectoderm at approximately E9.0. However, by E10.5, these genes are differentially expressed in neuronal progenitors in the developing OE.

To examine whether there is a defect that occurs in the formation of preplacodal region in *Six1;Six4* double mutant embryos, we analyzed the expression of *Eya2*. As shown in Fig. 3, its expression appeared normal in the double mutant at E8.0–8.5 (Fig. 3L), indicating that the preplacodal region was initially established but failed to develop into a normal olfactory placode. Thus, Six1/4 are likely to regulate the patterning of preplacodal ectoderm into an olfactory placode.

Altered expression of transcription factors in the presumptive olfactory ectoderm of Six1;Six4 double mutant embryos

We next analyzed the olfactory development both morphologically and by using molecular markers at E9.0 to 10.5. The homeobox gene *Dlx5* is expressed early in a broad domain in the ectoderm and is likely to regulate the expression of placode specific genes (Streit, 2007).

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At E9.25, *Dlx5* is normally expressed in the olfactory placode (Fig. 4A), and its expression appeared to be normal in *Six1*$^{-/-}$ embryos (Fig. 4B). However, in the double mutant embryos, *Dlx5* expression was markedly reduced in the presumptive olfactory ectoderm (arrow, Fig. 4C). To further confirm the defective formation of the olfactory placode in the double mutant embryos, we analyzed the expression of *Eya2* at E9.5–E10.5. *Eya2* is expressed in the specified olfactory placode (Fig. 4D) and its expression appeared to be unaffected in *Six1*$^{-/-}$ embryos (Fig. 4E). However, *Eya2* expression was detectable in the presumptive olfactory ectoderm but was largely reduced at E9.5 (arrow, Fig. 4F), in contrast to its normal expression in E8.5 double mutant embryos (Fig. 3K). Otx genes are expressed early in the preplacodal domain and in individual placodes (Schlosser, 2006). At E9.5, *Otx1* is strongly expressed in the olfactory placode (Fig. 4G) and its expression appeared normal in *Six1*$^{-/-}$ embryos (Fig. 4H). In *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos, its expression was also detectable in the malformed and slightly thickened epithelium (arrow, Fig. 4I). *Sox2* is not expressed in the early preplacodal region; it is expressed in the distinct lens, olfactory and otic placode (Kamachi et al.,1998; Wood and Episkopou, 1999). Similarly to *Six1, Sox2* expression was observed in the entire placode at around E9.75 (Fig. 4J) and its expression also appeared to be unaffected in *Six1*$^{-/-}$ embryos at this early stage (Fig. 4K). In contrast, in the double mutant embryos, *Sox2* expression was very faint in the malformed placodal epithelium at E9.75 (arrow, Fig. 4L) and became undetectable at E10.5 (data not shown). It was suggested that *Sox2* may act upstream of and interact with *Pax6* in lens and nasal placodal development (Donner et al., 2007). *Pax6* is expressed in the olfactory placode and plays an essential role in olfactory development (Fig. 4M; Donner et al., 2007 Grindley et al., 1995). In contrast, *Pax6* expression in the olfactory placode appeared to be reduced in *Six1*$^{-/-}$ embryos (Fig. 4N), and only residual expression was observed in *Six1*$^{-/-}$*;Six4*$^{-/-}$ embryos (arrow, Fig. 4O). Taken together, these results show that the ectoderm in the presumptive olfactory region in the double mutant appears to be induced for an olfactory fate as judged by the expression of molecular markers and the slight thickening of the ectoderm, but the abnormal morphology and altered expression of olfactory marker genes suggest that correct induction of the olfactory placode requires both *Six1* and *Six4*.

Altered expression of Fgfs and Bmp4 in the olfactory placode of Six1$^{-/-}$ and Six1$^{-/-}$;Six4$^{-/-}$ embryos

Members of Fgf and Bmp families are expressed during midfacial development and play diverse roles in induction and patterning of embryonic ectoderm. We next examined whether the expression patterns of *Fgf8, Fgf3, Fgf10* and *Bmp4* are normal during olfactory placode induction in embryos lacking *Six1* alone or both *Six1* and *Six4*. *Fgf8* is expressed in cells within a domain that encompasses a ring of ectodermal epithelium at the rim of olfactory pit (Fig. 5A) as well as adjacent neuroepithelial cells inside the pit (arrow, Fig. 5D). Inactivation of *Fgf8* results in an absence of OE, VNO, nasal cavity, forebrain, low jaw, eyelids, and pinnae (Kawauchi et al., 2005). In *Six1*$^{-/-}$ embryos, *Fgf8* expression domain in both the medial and lateral regions appeared to be expanded centrally when compared with controls (arrows, Figs. 5B, E). In contrast, *Six1*$^{-/-}$*;Six4*$^{-/-}$ embryos had weak *Fgf8* expression in the medial region (arrows, Figs. 5C, F). *Fgf10* is expressed in the dorsolateral region of the placode at E9.5 (data not shown; Bachler and Neubüser, 2001) and in the lateral region of the pit (Fig. 5G). Interestingly, *Fgf10* expression domain also appeared to be extended

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centrally in *Six1*$^{-/-}$ embryos (arrow, Fig. 5H). No *Fgf10* expression was detected in *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos (arrow, Fig. 5I). *Fgf3* is expressed in the peripheral domain of the medial side of the pit (Fig. 5J), next to the *Fgf8* domain (Fig. 5A). In *Six1*$^{-/-}$ embryos, *Fgf3* expression extended centrally in the pit (Fig. 5K) and was undetectable in *Six1;Six4* double mutant embryos (Fig. 5L). *Bmp4* has recently been shown to be required for olfactory placode induction (Sjödal et al., 2007). It is expressed in olfactory placode and pit (Figs. 5M, P and data not shown), and its expression appeared normal in *Six1*$^{-/-}$ embryos (Figs. 5N, Q). In *Six1;Six4* double mutant embryos, its expression was detectable at a reduced level at E9.5 (Fig. 5O) but was markedly reduced by E10.5 (Fig. 5R). The abnormal morphology and absent or altered expression domains of these signaling molecules in the mutant embryos suggest that there is likely a defect in placode induction in *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos and a defect in OE patterning in *Six1*$^{-/-}$ embryos. Our results indicate that both *Six1* and *Six4* may act synergistically to mediate the specification and patterning of olfactory placode through Fgf and Bmp signaling pathways.

All neuronal lineage markers are initially expressed but the pattern of neurogenesis is altered in Six1$^{-/-}$ embryos

A recent study has shown that loss of *Six1* function results in defective OE neurogenesis (Ikeda et al., 2007). We found that all neuronal markers are expressed in the mutant olfactory placode at E9.5, before the initiation of neurogenesis (Fig. 4). However, it remains unclear whether the pattern of neurogenesis is initiated normally. Here, we analyzed the patterns of *Six1-lacZ* and *Sox2* expression in control and mutant embryos at later stages respectively. X-gal staining of *Six1*^{lacZ} (*Six1*$^{+/-}$) embryos revealed high distribution of *Six1-lacZ* expression in the peripheral domains of the olfactory pit (Figs. 6A, B). In contrast, *Six1-lacZ* is expressed almost uniformly in the pit of *Six1*$^{lacZ/lacZ}$ (*Six1*$^{-/-}$) homozygous embryos (Figs. 6A', B'). At E12.0–12.5, *lacZ*-positive cells became localized in the basal progenitors (Supplemental Fig. 1). In contrast, in *Six1*$^{-/-}$ OE, *lacZ*-positive cells were observed in the recess region but no basal progenitors expressed *Six1-lacZ* by this stage (Supplementary Fig. 1). Similar to *Six1, Sox2* is expressed in the entire neuroepithelium at E10.5 but stronger in the peripheral progenitors (Kawauchi et al., 2005) (Figs. 6C, D). In the mutant embryos, *Sox2* transcripts were uniformly distributed throughout the OE at reduced levels (Figs. 6C', D'). At E12.5, it is expressed in both the basal and apical progenitors of the OE but no expression was observed in *Six1*$^{-/-}$ embryos (Supplemental Fig. 1). These observations suggest that the olfactory neural lineage was initially specified in *Six1*$^{-/-}$ embryos but they failed to undergo normal neural development. Consistent with this and previous observations (Ikeda et al., 2007), all neuronal lineage markers, including *Mash1, Ngn1, NeuroD, PhD1, Lhx2, Ebf1* and *Gap43* were expressed but their expression was severely affected in the mutant OE at E10.5 and completely lost at E12.5 (Figs. 6E–H, E'–H', Supplemental Fig. 2 and Table 1). In contrast to the previous observation that the pioneer neurons are absent in *Six1* mutant (Ikeda et al., 2007), very few pioneer neurons expressing *Ebf1* or *Lhx2* were detected in the mutant embryos at E10.5 (arrows, Figs. 6E–H, E'–H' and Table 1). As we have not observed *Six1* expression in terminally differentiated OSNs and neurogenesis appears to have ceased by E12.5, *Six1* is likely to control OSN differentiation program by regulating the expansion of neural progenitor cells during primary neurogenesis.

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Increased apoptosis and reduced proliferation in the OE of Six1$^{-/-}$ embryos

It was reported that dysgenesis of the OE in *Six1$^{-/-}$* embryos is not due to increased apoptosis or defective cell proliferation, as neither abnormal apoptosis nor reduced cell proliferation in the OE was observed in the mutant, although increased cell death was observed in the mesenchyme surrounding the OE (Ikeda et al., 2007). Because we failed to detect any visible OE in older *Six1$^{-/-}$* embryos on histological sections, it is possible that precursor cells observed in the younger mutant embryos degenerate and thus fail to form a morphologically detectable OE. We therefore set out to determine whether the precursor cells in the mutant OE undergo abnormal cell death. Coronal sections of E9.5 to 12.5 embryos were processed for the TUNEL detection method of apoptotic nuclei. At E9.5, abnormal apoptosis was not observed in either single or double mutant embryos (Figs. 7A–C, I). It should be noted that the total number of epithelial cells in the thickened placodal region in the double mutant is largely reduced when compared with control or *Six1* single mutant (Figs. 7A–C). By E10.5, more apoptotic cells in the peripheral domains of invaginating placode were detected in *Six1$^{-/-}$* embryos (arrows, Figs. 7E, I) when compared with controls at E10.5 (Fig. 7D). However, the number of apoptotic cells was not increased in the double mutant at E10.5 when compared with E9.5 (Figs. 7F, I). At E12.5, apoptotic cells throughout the OE were apparent in the mutant (arrows, Figs. 7H, I), whereas very few apoptotic cells were seen in the controls (Fig. 7G). The apoptotic cells detected in the mutant OE were localized within the Six1 expression domain (Fig. 1 and data not shown). At E13.5, massive cell death was evident and no morphological detectable OE was present in the mutant (data not shown). In addition to the epithelium, apoptotic cells were present in the mesenchyme (Figs. 7D–H), but no obvious difference was observed between control and mutant embryos. Thus, the degeneration of *Six1$^{-/-}$* OE can be attributed, at least in part, to increased cell death of the epithelium.

We next sought to confirm whether *Six1$^{-/-}$* olfactory neuronal precursor cells proliferate appropriately by assaying BrdU incorporation in the mutant epithelium at E9.5, 10.0 and 12.0, before apparent cell apoptosis was seen in *Six1$^{-/-}$* embryos. Four hours after BrdU injection, BrdU-labeled cells were seen throughout the olfactory placode but were dense in the basal region in control embryos at E9.5–10.0 (Fig. 8A). In contrast, the number of BrdU-labeled cells in *Six1$^{-/-}$* embryos was slightly reduced in the placode at E9.5 (Fig. 8B) but more reduced at E10.5 when compared with controls (Fig. 8E). However, in *Six1$^{-/-}$;Six4$^{-/-}$* embryos, the number of BrdU-labeled cells in the placodal region was markedly reduced when compared with controls (Figs. 8C, F). At E12.0, BrdU-positive cells were dense in both basal and apical layers of the OE (Fig. 8G). In contrast, BrdU-positive cells were largely reduced in *Six1$^{-/-}$* OE at E12.0 and the mutant OE is not well patterned into basal, intermediate and apical layers (Figs. 8H, I). Thus, *Six1* is likely to regulate normal expansion of neuronal progenitors and their patterning in the OE by controlling their proliferation during early neurogenesis. Furthermore, these results demonstrate that *Six1* and *Six4* have a synergistic role in regulating epithelial cell proliferation during placode formation, uncovering previously unknown function of these genes during OE development.

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Discussion

The olfactory placode is derived from a common preplacodal domain around the neural plate. The common primordium expresses members of the *Six–Eya–Dach* genes in *Xenopus*, zebrafish, and chick (Schlosser, 2006; Brugmann and Moody, 2005; Streit, 2004). Within the preplacodal region, precursors for different placodes are initially interspersed, but then separate to form individual placodes by thickening the epithelium at discrete positions (Kozlowski et al., 1997; Streit, 2004; Bhattacharyya et al., 2004). It has been proposed that the olfactory placode develops through the anterior convergence of the cells in the olfactory field (Whitlock and Westerfield, 2000). However, the molecular events that initiate the transition from preplacode to placode stages and the function of Eya–Six in the preplacodal domain are essentially unknown. In this study, we show that *Six1/4* and *Eya2* are coexpressed in the preplacodal ectoderm in mouse embryos and their expression is subsequently restricted to individual placodes, including the olfactory placode. Therefore, the *Eya–Six* genes may have an evolutionarily conserved function in regulating the properties of preplacodal ectoderm and its transition to morphologically individual placodes.

Our results show a synergistic requirement of *Six1* and *Six4* in patterning the presumptive placodal ectoderm to a morphological placode. In *Six1;Six4* double mutant embryos, the preplacodal region is initially formed as labeled by *Eya2* expression but the olfactory placode was severely malformed. In addition, the mutant ectoderm failed to invaginate in all cases examined and this phenotype was fully penetrant. However, based on its slightly thickened appearance between E9.5 and 10.0, the mutant ectoderm was likely induced for an olfactory fate.

Fgf and Bmp signals from neighboring tissues induce placode formation and activate specific patterns of gene expression (Wilson et al., 2001; Bailey et al., 2006; Sjödal et al., 2007). Olfactory placode induction has been shown to involve Bmp and Fgf signals and their temporal and spatial integration is important to generate placode precursors (Sjödal et al., 2007). In *Xenopus* embryos, a combination of Fgf8 and low levels of Bmp activity induces *Six1* expression in placodal progenitor cells (Ahrens and Schlosser, 2005). These signaling molecules are also expressed during olfactory placodal development and previous studies have suggested a role for Fgf8 in maintenance or proliferation rather than in specification of olfactory placodal cells (Kawauchi et al., 2005). However, it is unclear whether Bmp signals are required for the differentiation of placodal cells after their initial specification as *Bmp4*$^{-/-}$ mice develop normal olfactory placodes (Furuta and Hogan, 1998). In the present study, we found that the maintenance of *Bmp4* expression in the placodal epithelium requires Six1 and Six4 function. This observation raises the possibility that both Six1 and Six4 may regulate normal patterning, morphogenesis or cellular differentiation of olfactory placode through Bmp4-signaling pathway.

Fgf3 and Fgf10 have been shown to play an essential role in otic placode induction (Wright and Mansour, 2003), while Fgf3, Fgf8 and Fgf10 also show differential requirements during inner ear development (Zelarayan et al., 2007). However, it is currently unclear whether Fgf3 and Fgf10 play any role in olfactory placode induction and its subsequent growth. As we found that normal expression of these Fgf signaling molecules in the olfactory

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epithelium is *Six1/4*-dependent, it is plausible that Six1/4 regulate Fgf signals that stimulate placode formation and promote its normal growth. Detailed examination of olfactory development in *Fgf3, Fgf10* and *Fgf8* double or triple mutant will provide insights into the regulatory relationships between Six1/4 and Fgfs and how these Fgfs act to differentially regulate the patterning of the olfactory system.

Many transcription factors are expressed in the preplacodal region and previous studies have suggested that they work together to define each specific placode (Whitlock, 2004). Dlx genes are expressed early in preplacodal ectoderm and implicated in promoting placodal competence (Long et al., 2003). Otx genes are also expressed in individual placodes from very early stages and they may act as positional markers for placode identity. Sox family transcription factors are expressed in specified placodes and are involved in promoting neuronal progenitors, while the bHLH transcription factors Mash1 and Ngn, the POU domain and other transcription factors are involved in promoting cytodifferentiation of various placodal cell types. Our data show that the expression of *Dlx5*, Otx and *Sox2* genes were detectable in the presumptive olfactory ectoderm of the double mutant embryos, but we failed to detect the expression of transcription factors promoting neuronal differentiation, including *Mash1* and *Ngn1* (data not shown). Based on these results, we speculate that the weak expression of *Dlx5*, Otx and *Sox2* in the double mutant ectoderm may respond to the inductive signals to induce partial thickening of the ectoderm. Six1/4 may cooperate with Dlx5, Otx and Sox2 as well as other transcription factors to activate the olfactory program. Six1/4 may also interact with Sox2 and other transcription factors to specify a subset of epithelial cells in the placode to acquire a neuronal cell fate, and the *Sox2*-expressing cells present in the double mutant ectoderm are uncommitted epithelial cells. This explains why the double mutant ectoderm failed to express any early olfactory neuronal lineage markers (data not shown). Our analyses suggest that the threshold of Six1/4 appears to be crucial for the regulation of olfactory specific gene expression as we detected that the expression of many genes in the presumptive olfactory ectoderm was further reduced in the double mutant embryos.

The olfactory ectoderm is programmed for neurogenesis from as early a stage as when the olfactory placode becomes morphologically apparent. The neuroblast precursors normally undergo committed neuronal differentiation to form the OSNs and neurogenesis occurs in a periphery-to-center pattern that reflects the stage of each expressing cell in the neuronal lineage. As our results suggest a correlation between *Six4* expression and initiation of neuronal differentiation in the OE, it is tempting to speculate that *Six4* may also specify neuronal phenotype. Although *Six4* mutant mice appear normal, *Six1* and *Six4* may function synergistically in initial neuronal commitment. This explains why the neurogenesis is initiated in an abnormal pattern in *Six1*$^{-/-}$ embryos. However, our results clearly show that *Six4* cannot functionally compensate for the loss of *Six1*.

We compared the expression of each neuronal lineage marker in control and *Six1*$^{-/-}$ OE and our results show that loss of *Six1* results in not only a reduction in the number of cells expressing each marker but also an alteration in their spatial distribution, which has not been previously noted. The uniform *Sox2* expression throughout the epithelium in *Six1*$^{-/-}$ embryos suggests that some of the *Sox2*-expressing stem cells located at the peripheral

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domains were already lost in the mutant at E10.5. The olfactory neurogenesis in *Six1*$^{-/-}$ embryos does not appear to occur in a periphery-to-center pattern, as *Mash1*-expressing cells, which mark the earliest committed neuronal progenitors, were distributed throughout the invaginating olfactory pit. The mutant ectodermal cells that already committed to neuronal lineage were able to continue their differentiation program and become immature neurons, but the population of neuronal precursors was significantly reduced. One likely explanation for the reduction of neurogenesis is that *Six1* is required for expansion of neuronal progenitors. Consistent with this idea, BrdU incorporation studies revealed fewer BrdU-labeled cells in the olfactory placode in *Six1* single as well as *Six1;Six4* double mutants (Fig. 8). In addition, more TUNEL-positive cells were detected in the OE of *Six1*$^{-/-}$ mutant than in normal embryos. Thus, enhanced apoptotic cell death may lead to the failure of establishing the mature characteristics of the OE in *Six1*$^{-/-}$ mutant.

It should be mentioned that analysis of null mutations in *Six1* by two groups resulted in controversy over whether the pioneer neurons were initially present in the mutants and whether the mutant neuronal progenitors underwent abnormal apoptosis or failed to proliferate normally. These discrepancies may be caused by the nature of two distinct *Six1* mutations. However, given the limited number of pioneer neurons labeled by *Ebf1* or *Lhx2* in the mutant (Table 1), they can easily be missed on sections due to several factors, including slight differences in the thickness of sections and the stages of the embryos, an increase in apoptosis, and a reduction of proliferation of neuronal progenitors during differentiation. This also explains why no pioneer neurons expressing the terminal differentiation marker *Gap43* were observed (Supplemental Fig. 2 and Table 1). Our findings of reduced cell proliferation and increased apoptosis in the mutant OE are consistent with previous observations of *Six1* regulating cell proliferation and cell survival in other developmental systems, including the otic placode, kidney mesenchyme, and pharyngeal endoderm (Xu et al., 2003; Zheng et al., 2003; Zou et al., 2006a,b). Thus, *Six1* may function as a regulator of cell differentiation, and loss of *Six1* will lead to apoptosis due to a failure of normal cellular specification.

Although the molecular details of how Six1 acts to regulate cell proliferation and survival are unclear, the observation of Six1 being highly distributed in the peripheral stem cells suggests that Six1 may act together with Sox2 to specify the neuronal progenitors and to initiate olfactory neuronal cell differentiation program by regulating downstream transcription factor Mash1. Indeed, we found that the two proteins physically interact and form a complex (data not shown). It will be important to determine whether Six1/4 have essential functions and are upstream regulators for *Sox2* or function in parallel with Sox2 in the generation of neuronal progenitor cells in the OE. *Six1* may also interact with *Fgf* signaling to directly regulate the expression of genes that are involved in cell proliferation and survival. Interestingly, we found that the *Six4*-positive cells are grouped into clusters, similar to the *Mash1* domains. Therefore, it is also possible that Six4 may partially compensate for the loss of Six1 function and the threshold of Six1/4 is critical to initiate neuronal differentiation program by activating or interacting with Mash1. Additional analysis will be required to elucidate their precise mode of action in olfactory neuronal cell lineages.

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We have previously shown that the *Eya1–Six1* regulatory cassette is conserved in several developmental systems and mutations in both genes in humans cause branchio-oto-renal (BOR) syndrome (Xu et al., 2003; Zheng et al., 2003; Ruf et al., 2004; Zou et al., 2004, 2006a,b; Sajithlal et al., 2005). In the present study, we found that *Eya1* expression in the placodal ectoderm is turned on after *Eya2, Six1* and *Six4* are already expressed. In the olfactory pit, *Eya1* is not expressed in the stem cells located at the peripheral regions where *Eya2* and *Six1* are strongly expressed. $Eya1^{-/-}$ mice show normal development of the olfactory system, while $Eya2^{-/-}$ mice do exhibit a later phenotype during OE neurogenesis (B. Chen, E-H. Kim and P-X. Xu, manuscript in preparation). Interestingly, *Eya1* and *Eya2* also appear to function redundantly during OE neurogenesis as *Eya1;Eya2* double mutant display more severe phenotype than each single mutant (B. Chen, E-H. Kim and P-X. Xu, manuscript in preparation). However, the phenotype observed in *Eya1;Eya2* double mutant is less severe than seen in *Six1; Six4* double mutant. This could be due to functional redundancy with other members of the Eya gene family. Indeed, we found that *Eya4* is also expressed early in the olfactory placode ectoderm (data not shown) (Borsani et al., 1999). This could explain why the olfactory abnormalities in *Eya1;Eya2* occur later. As *Eya2* expression was unaffected in *Six1;Six4* double mutant ectoderm and showed overlapping pattern with *Six1* domain (Figs. 1 and 3), the Eya–Six regulatory cassette is likely to operate during olfactory development. Analysis of *Eya2;Eya4* double mutant embryos and the expression of Six gene family in those mutant at placodal stages should further clarify the regulatory relationship between Eya and Six genes during the initiation of olfactory development.

Our observation of downregulation of *Pax6* expression in *Six1;Six4* double mutant raised questions regarding the relationship between the Pax and Six genes. In *Drosophila, eyeless/Pax6* acts upstream of *eya* and *so* but a regulatory feedback is required to maintain the expression of all three genes (Bonini et al., 1997; Halder et al., 1998). Although we cannot exclude the possibility that *Six1/4* act genetically upstream of *Pax6* during early olfactory placode formation, *Six1* and *Six4* may participate in a positive feedback loop to upregulate *Pax6* expression in the ectoderm and in the absence of *Six1/4, Pax6* expression is not fully activated or maintained. Consistent with this view, we found that *Pax2* expression was markedly reduced in $Six1^{-/-}$ metanephric kidney mesenchyme (Xu et al., 2003). Since olfactory placode formation was also severely affected in the *Pax6* mutant embryos (Grindley et al., 1995), early events of olfactory development may not be triggered by a simple linear pathway but by a complex regulatory network of gene activities involving Pax, Eya and Six. Nonetheless, our results suggest that the *Six1/4* control critical early inductive events that are required for the olfactory development and they may act together with *Sox2* to activate OSN differentiation program in the OE.

Acknowledgments

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Appendix A. Supplementary data

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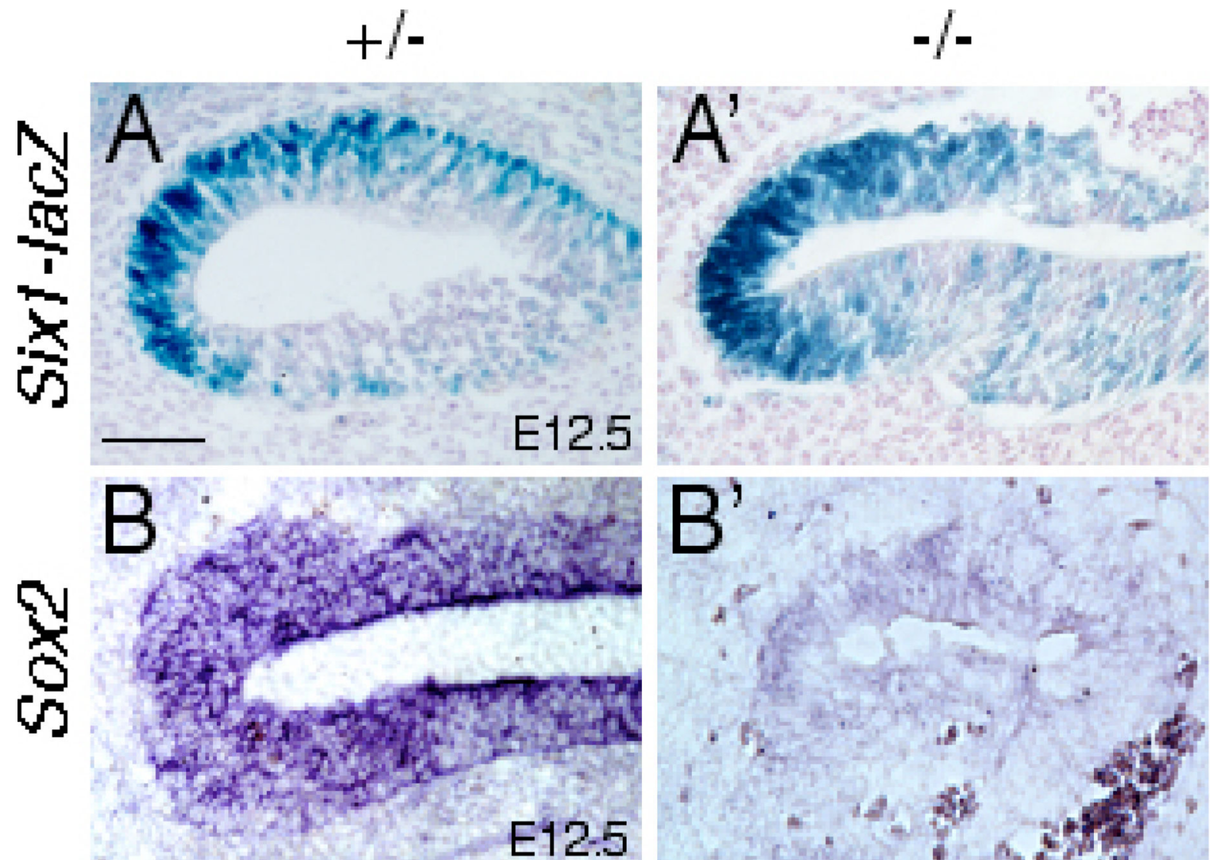

Supplemental Fig. 1.

Neurogenesis ceases at E12.5 in *Six1*$^{-/-}$ embroys. (A,A') Sections of X-gal stained *Six1*$^{lacZ/+}$ (*Six1*$^{+/-}$) or *Six1*$^{lacZ/lacZ}$ (*Six1*$^{-/-}$) heads showing *Six1-lacZ* expression in the developing OE at E12.5. (B,B') Section in situ showing *Sox2* expression in *Six1*$^{+/-}$ or *Six1*$^{-/-}$ OE. Scale bar: 50 μm.

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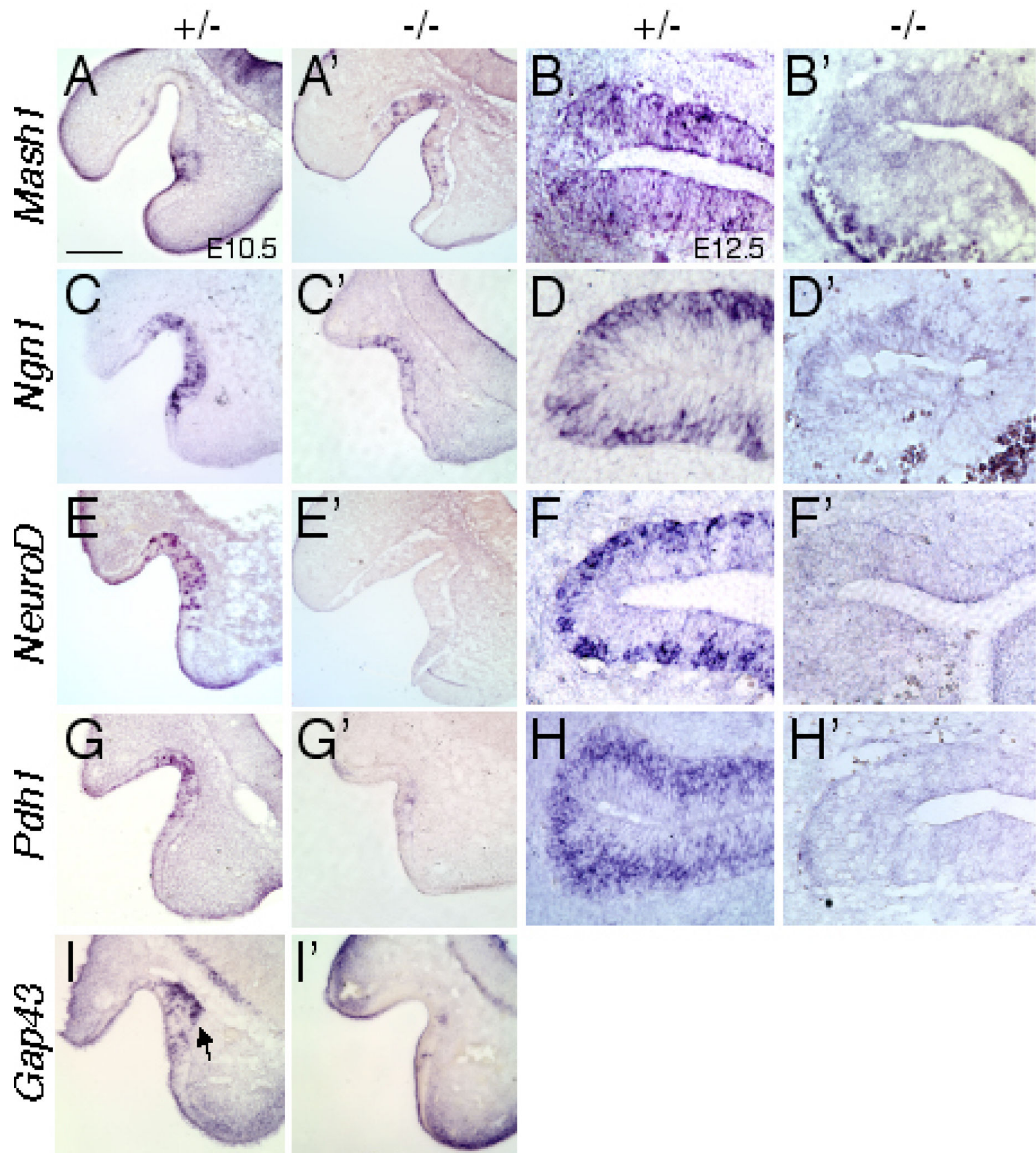

Supplemental Fig. 2.

All neuronal lineage markers are initially expressed but disappear by E12.5. Section in situ hybridization of Mash1, Ngn1, NeuroD, Pdh1 and Gap43 in control and Six1 mutant embryos at E10.5 and 12.5. Arrow in I points to pioneer neurons. For all panels, sections are coronal and rostral is up. Scale bars: 100 μm.

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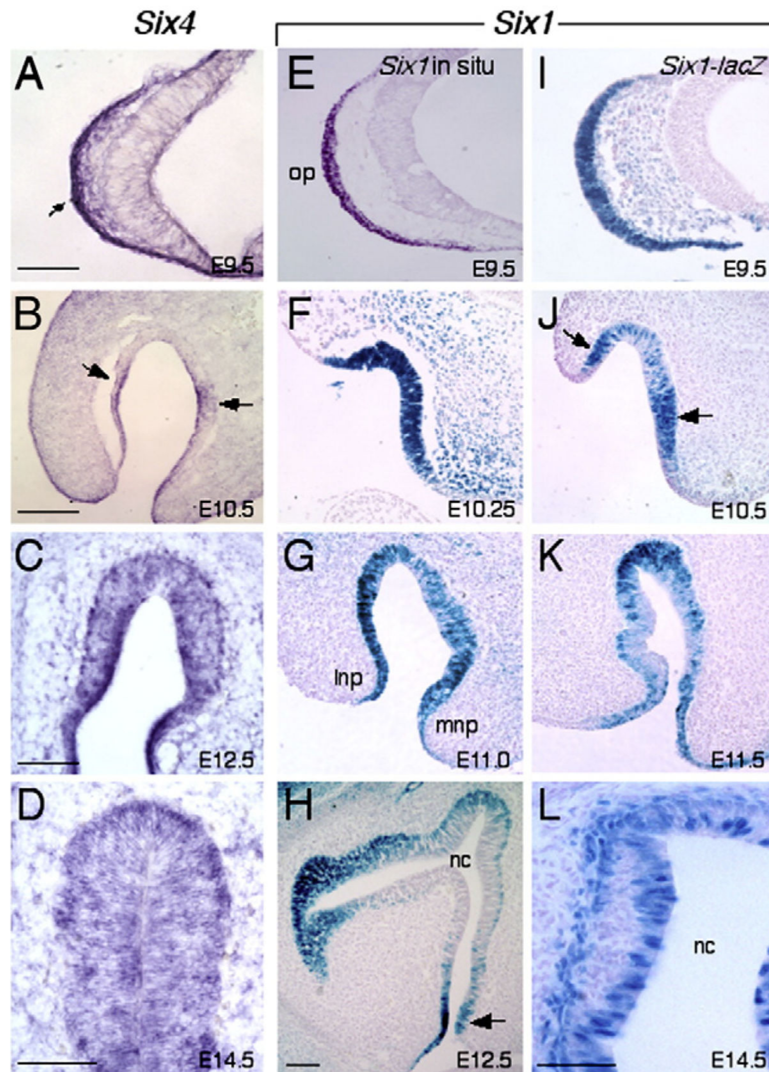

Fig. 1.

Six1 is expressed in the neuronal progenitors of the olfactory epithelium. (A–D) Section in situ hybridization showing *Six4* expression in a wide region in the presumptive olfactory ectodermal region at E9.5 (arrow in panel A), and (B) its expression overlaps with *Mash1* domains at E10.5 (arrows). (G, J) *Six4* expression in the developing OE at E12.5 (C) and 14.5 (D). (E) Section in situ hybridization and (I) sections of X-gal stained *Six1*$^{lacZ/+}$ embryos showing *Six1* expression in a large domain of the olfactory placodal (op) region at E9.5. (F–H, J–L) Sections of X-gal stained *Six1*$^{lacZ/+}$ embryos showing *Six1-lacZ* expression in the invaginating olfactory pit at E10.25–11.5 and in the OE at E12.5 and 14.5.Arrows in panel J point to the higher distribution of *Six1* in the periphery regions of the olfactory epithelium. Arrow in panel L points to strong *Six1* expression in the ectoderm near the nasopharynx. Abb.: lnp, lateral nasal process; mnp, medial nasal process; nc, nasal cavity. For all panels, sections are coronal and rostral is up. Scale bars: 100 μm.

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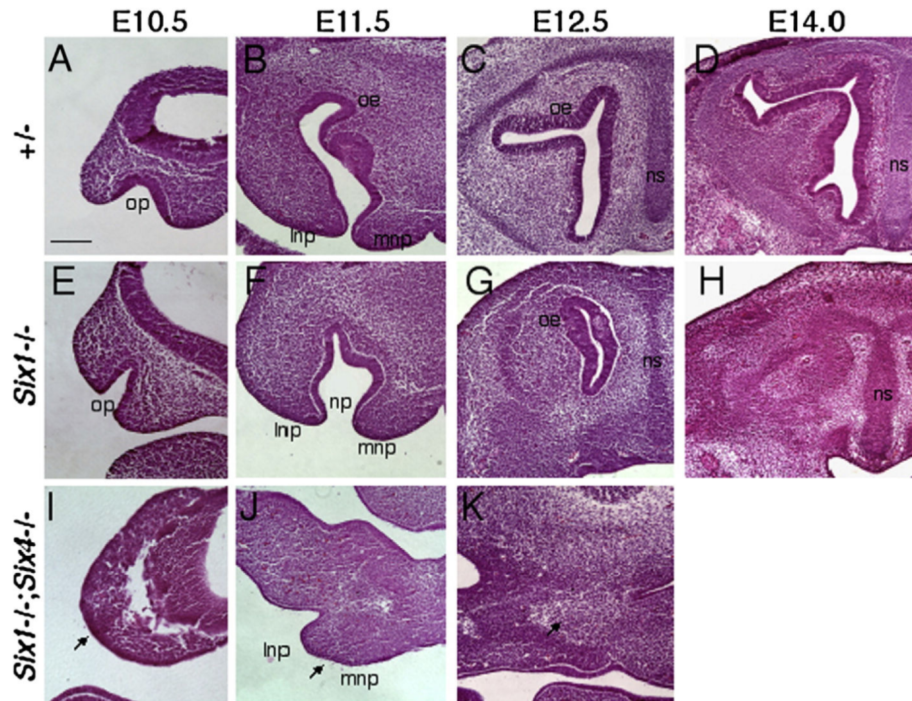

Fig. 2.

Histological analysis of *Six1* single and *Six1;Six4* double mutant olfactory development. (A–K) H&E stained coronal sections showing the nasal region of control (A–D), *Six1*$^{-/-}$ (E–H) and *Six1*$^{-/-}$;*Six4*$^{-/-}$ mutant (I–K) embryos from E10.5 to E14.5. Arrows point to the presumptive nasal region in the double mutant embryos. Abb.: op, olfactory pit; lnp, lateral nasal process; mnp, medial nasal process; ns, nasal septum; oe, olfactory epithelium; VNO, vomeronasal organ. For all panels, rostral is up. Scale bar: 100 μm.

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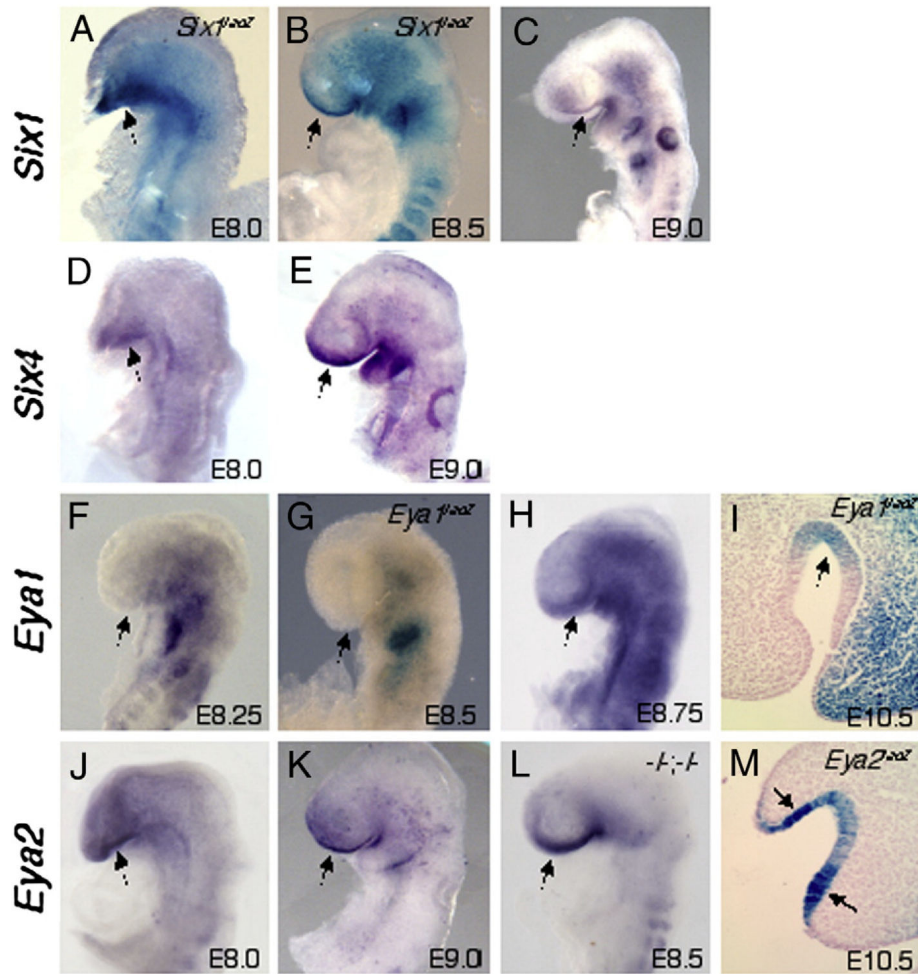

Fig. 3.

Analysis of *Six1, Six4, Eya1*, and *Eya2* expression in the placodal ectoderm from E8.0–10.5. (A, B) X-gal staining of *Six1*^{lacZ} embryos showing *Six1-lacZ* expression in the preplacodal region at E8.0 and E8.5 (arrows) and (C) in situ hybridization showing *Six1* expression in the presumptive olfactory ectoderm at E9.0 (arrow). (D, E) In situ hybridization showing *Six4* expression in the preplacodal region at E8.0 (arrow in panel D) and in the olfactory ectoderm at E9.0 (arrow in panel E). (F, H) In situ hybridization and (G, I) X-gal staining of *Eya1*^{lacZ} embryos showing that *Eya1* is not expressed in the preplacodal region at E8.25–8.5 (arrow in panels F, G) but its expression became detectable at around E8.75 (arrow in panel H). Arrow in panel I points to *Eya1-lacZ*-expressing cells in the center of olfactory pit at E10.5. (J, K) In situ hybridization showing *Eya2* expression in the preplacodal ectoderm (J) and in the olfactory ectoderm at E9.0 (arrows) in control embryos, and (L) in *Six1;Six4* double mutant embryos at E8.5 (arrow). (M) At E10.5, X-gal staining of *Eya2*^{lacZ} showing its expression strongly in the peripheral progenitors (arrows) and weakly in the center domain. For panels I and M, sections are coronal and rostral is up.

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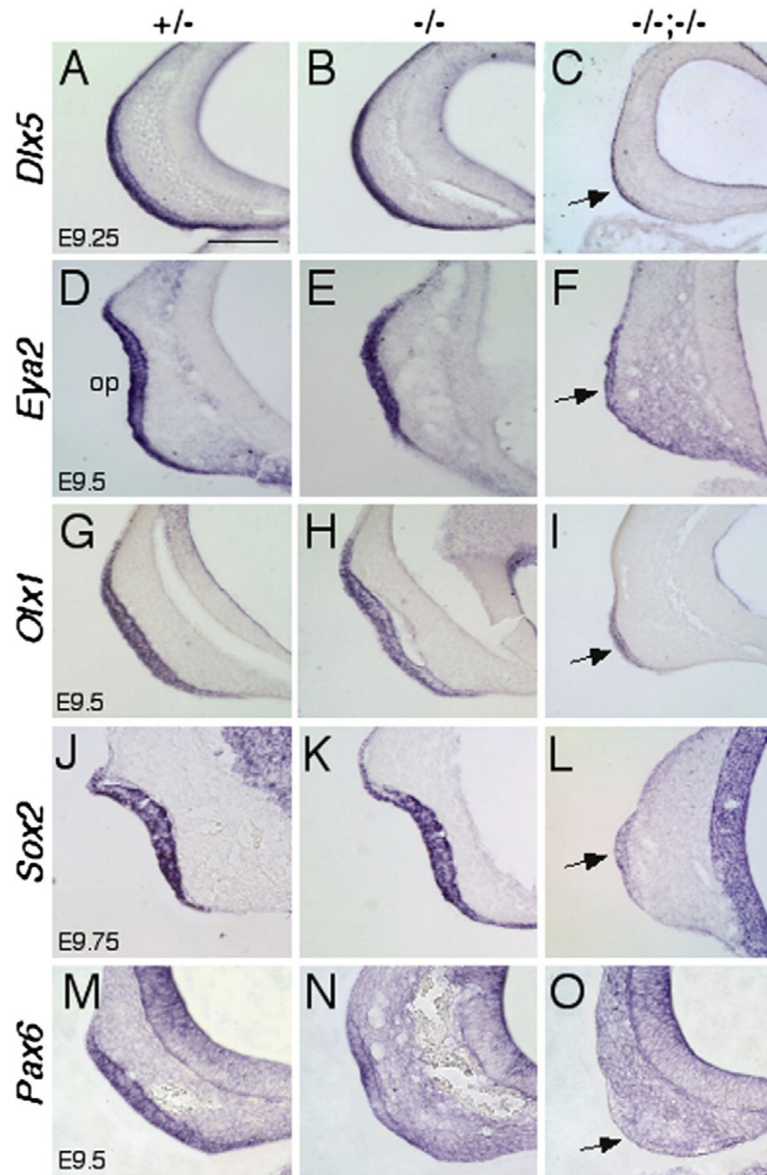

Fig. 4.

Failure of olfactory placode formation in *Six1;Six4* double mutant embryos. In situ hybridization on coronal sections through the nasal region of E9.5–E9.75 *Six1*$^{+/-}$, *Six1*$^{-/-}$ single or *Six1*$^{-/-}$;*Six4*$^{-/-}$ double mutant embryos showing *Dlx5, Eya2, Otx1, Sox2* and *Pax6* expression in the olfactory ectoderm. Arrows in panels C, F, I, L, O point to presumptive olfactory regions in *Six1;Six4* double mutant embryos. For all panels, rostral is up. Scale bar: 100 μm.

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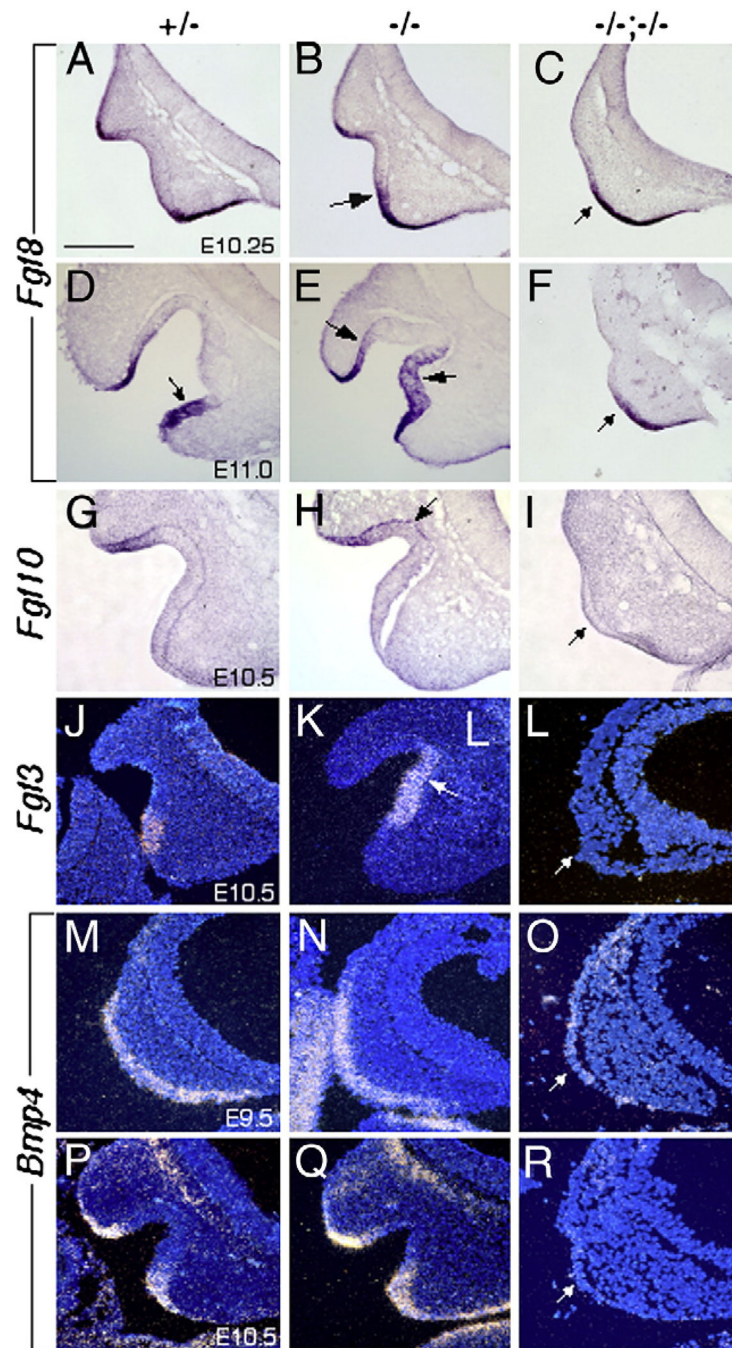

Fig. 5.

Altered *Fgf8, Fgf10, Fgf3* and *Bmp4* expression in *Six1*$^{-/-}$ and *Six1*$^{-/-}$*;Six4*$^{-/-}$ embryos. (A–F) Section in situ hybridization showing *Fgf8* expression in olfactory region in control, *Six1* single and *Six1;Six4* double mutant embryos. Arrows in panels B, E point to the central extension of *Fgf8* expression domain in the epithelium in *Six1*$^{-/-}$ embryos. Arrow in panel D points to *Fgf8* expression in the medial epithelium. Arrows in panels C, F point to the presumptive olfactory ectoderm region in the double mutant embryos. (G–I) Section in situ hybridization showing *Fgf10* expression in the lateral domain of the pit (G), its central

extension in *Six1*$^{-/-}$ embryos (arrow in panel H), and no expression in *Six1;Six4* double mutant (arrow). (J–L) Radioisotope in situ hybridization showing *Fgf3* expression in the peripheral domain in the medial pit (J), its central extension in *Six1*$^{-/-}$ embryos (arrow in panel K) and no expression in *Six1*$^{-/-}$*;Six4*$^{-/-}$ embryos (arrow in panel L). (M–R) Radioisotope in situ hybridization showing *Bmp4* expression in the olfactory placode at E9.5 and the pit at E10.5 in control and mutant embryos. For all panels, sections are coronal and rostral is up. Scale bars: 100 µm.

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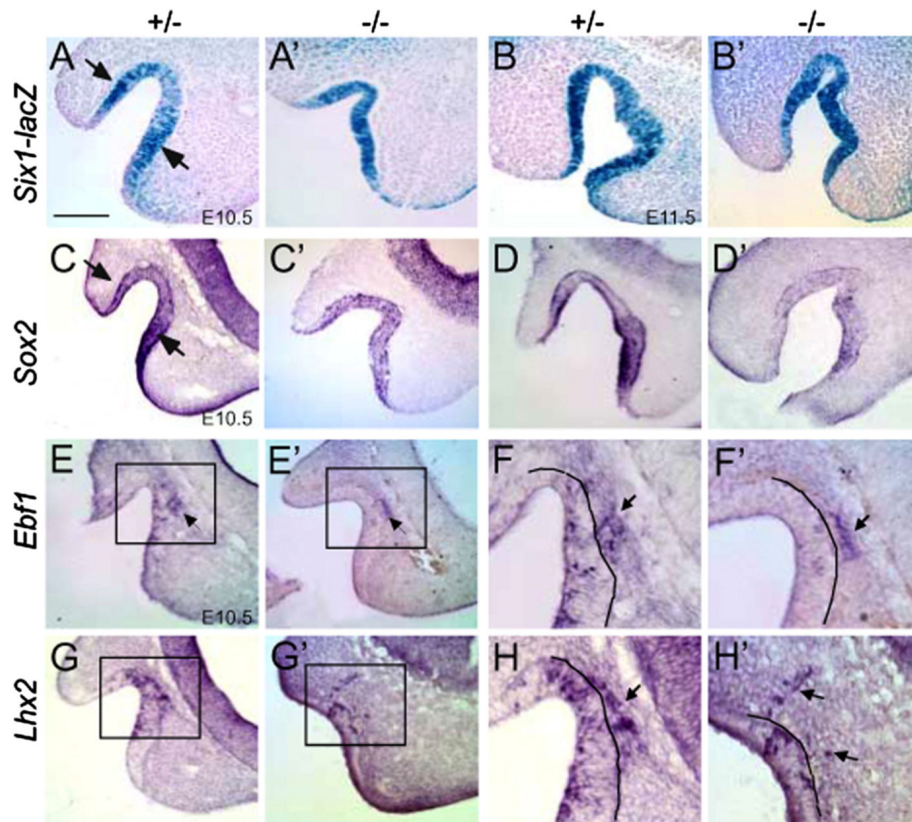

Fig. 6.

OE neurogenesis occurs in an abnormal pattern in *Six1*$^{-/-}$ embryos. (A, A', B, B') Coronal sections of X-gal stained *Six1*$^{lacZ/+}$ (*Six1*$^{+/-}$) or *Six1*$^{lacZ/lacZ}$ (*Six1*$^{-/-}$) heads showing *Six1-lacZ* expression in the developing OE at E10.5 and E11.5. Arrows in panel A point to the dense *Six1*-expressing cells in the peripheral regions of the epithelium. (C, C', D, D') Section in situ showing *Sox2* expression in *Six1*$^{+/-}$or *Six1*$^{-/-}$ OE. Arrows in panel C point to the strong *Sox2* expression in the peripheral regions of the epithelium (E–H, E'–H') Coronal section in situ hybridization showing *Ebf1* (E, E', F, F') and *Lhx2* (G, G', H, H') expression in control and *Six1* mutant embryos. Arrows point to the pioneer neurons migrated away from the epithelium. (F, F', H, H') Higher magnification of boxed areas in E, E', H and H' respectively. For all panels, rostral is up. Scale bar: 100 μm. 200 μm for panels F, F', H and H'.

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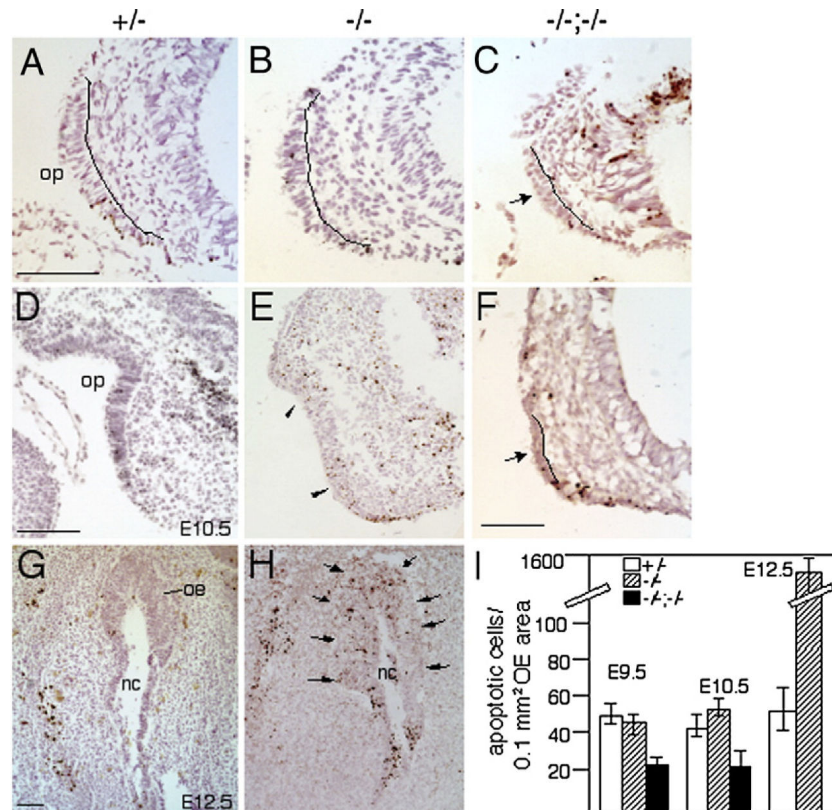

Fig. 7.

Six1$^{-/-}$ olfactory progenitor cells undergo abnormal apoptosis from E10.5. (A–H) TUNEL analysis of coronal sections through the nasal region of *Six1*$^{+/-}$ and *Six1*$^{-/-}$ at E9.5, 10.5 and 12.5 for labeling apoptotic bodies (brown staining). Arrows point to numerous apoptotic bodies detected in the mutant. nc, nasal cavity. For all panels, rostral is up. (I) Statistic analysis of apoptotic cells. Total apoptotic cells in OE were counted and data for each OE were summed and normalized to 0.1 mm^2, the average total OE area in a section at E12.5. Five embryos per genotype were counted; *P* values were calculated using StatView *t*-test. Error bars indicate standard deviation. Scale bars: 100 μm.

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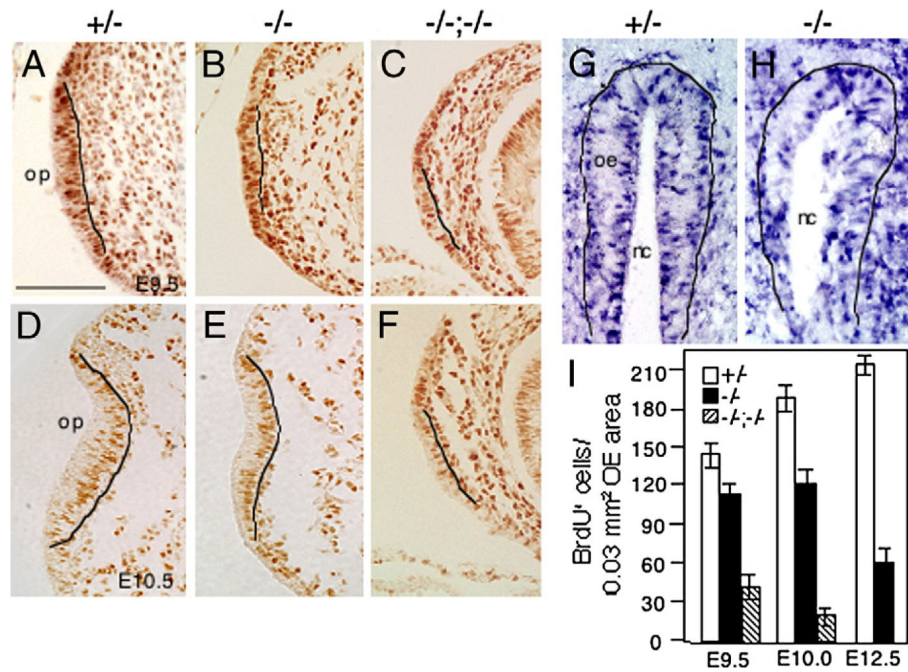

Fig. 8.

Six1 controls proliferation of olfactory progenitor cells during early olfactory development. Coronal sections of nasal regions from E9.5 to E12.0 *Six1*$^{+/-}$ (A, D, G), *Six1*$^{-/-}$ (B, E, H) and *Six1*$^{-/-}$;*Six4*$^{-/-}$ (C, F) embryos showing BrdU-labeled cells (brown or blue). nc, nasal cavity; oe, olfactory epithelium. For all panels, rostral is up. (I) Statistic analysis of BrdU-positive cells from each olfactory pit or OE. Data from each OE (minimal from 2 animals) were summed and normalized to 0.03 mm^2. *P* values were calculated using StatView *t*-test. Error bars indicate standard deviation. Scale bars: 100 μm.

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Table 1

Deficits in neuronal cell types in the OE and in pioneer neurons of Six1 mutants at E10.5

Neuronal lineage marker	Mean number of cells in the epithelium/olfactory pit (s.e.m.)		Number of pioneer neurons/olfactory pit (s.e.m.)		***n*** **(olfactory placodes examined)**	***p*** **(*t*-test)**
	Control	**Mutant**	**Control**	**Mutant**		
Mash1	238.75 (13.70)	125.25 (17.32)		Control = 5, mutant = 5	0.0257	
Ngn1	545.25 (13.97)	76.75 (10.82)		Control = 6, mutant = 6	0.0001	
Pdh1	128.67 (11.21)	15.5 (2.02)		Control = 4, mutant = 4	0.0001	
Lhx2	204.25 (12.24)	28 (9.29)	82.5 (13.90)	3.2 (1.20)	Control = 8, mutant = 4	0.0006
Ebf1	145.5 (13.26)	21.25 (1.49)	93.3 (14.70)	9.5 (1.12)	Control = 6, mutant = 4	0.0004
Gap43	127 (4.96)	10.75 (1.10)	123.5 (3.50)	0	Control = 4, mutant = 4	0.0001

In situ hybridization for each neuronal marker was performed on serial sections (15 μm) through the olfactory pit in E10.5 control and mutant littermates. The number of cells expressing a given marker and total OE area were measured for all sections encompassed by a given olfactory pit.